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13. ABSTRACT (Maximum 200 Words)

We have identified a group of synthetic triterpenoids, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and its derivative 1-(2-cyano-3,12-dioxooleana-1,9-dien-28-oyl) imidazole (CDDO-Im), which induce apoptosis in breast and prostate cancer cells. Moreover, sub-lethal doses (nanomolar range) of triterpenoids sensitize TRAIL-resistant breast and prostate cancer cells to TRAIL-mediated apoptosis. For example, in T47D and MDA-MB-468 breast cancer cells, TRAIL fails to initiate caspase-8 processing and consequently does not initiate TRAIL-mediated apoptosis. Concomitant treatment with CDDO or CDDO-Im reverses the TRAIL-resistant phenotype, leading to rapid induction of TRAIL-mediated apoptosis, while having no adverse effects on normal human mammary epithelial cells (HMEC). Mechanistically, CDDO and CDDO-Im 1) down-regulate the anti-apoptotic protein c-FLIP, which inhibits caspase-8 activation at the DISC (death-inducing signaling complex), 2) and induce up-regulation of the death receptors DR4 and DR5 on the cell surface. The combination of CDDO-Im and TRAIL reduces tumor burden in an in vivo MDA-MB-468 tumor xenograft model. After 14 days of combination CDDO-Im and TRAIL treatment, we found no significant toxicity in mouse tissues or hematological parameters. In conclusion, triterpenoids used either alone, or in combination with TRAIL, represent a promising new cancer therapy, deserving of further pre-clinical testing.

14. SUBJECT TERMS
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INTRODUCTION:

Our laboratory has begun to explore the possibility of exploiting biological response modifiers as agents for restoring apoptosis sensitivity to breast and prostate cancers. Among the agents studied in our lab are synthetic triterpenoids. Triterpenoids represent a class of naturally occurring and synthetic compounds with demonstrated anti-tumor activity. CDDO (2-Cyano-3,12-Dioxoolean-1,9-Dien-28-Oic Acid) [1] and two of its synthetic derivatives CDDO-Me (methyl ester) and CDDO-Im (imidazolide) [2] have recently been shown to induce apoptosis of malignant cells in vitro [3], and in vivo [4], [5]. Data from our laboratory indicate that these synthetic triterpenoids have the property of reducing expression of anti-apoptotic proteins that suppress apoptosis induction by TNF-family cytokines, including TRAIL – a promising biological response modifier which is soon to enter human clinical trials. One of the anti-apoptotic proteins modulated by these triterpenoids is FLIP, a potent inhibitor of death receptor-mediated apoptotic signaling [6].

Recently, we have found that CDDO and CDDO-Im induce apoptosis in breast and prostate cancer cells at doses in the 1-5 µM range (data shown below). In addition, we have also observed that CDDO and CDDO-Im, when used at sub-micromolar concentrations (generally sub-optimal for apoptosis induction) will sensitize TRAIL-resistant breast and prostate cancer cells to TRAIL-mediated apoptosis. One of our specific aims was to determine the mechanism(s) triterpenoids utilize to sensitize cancer cells to TRAIL-mediated apoptosis. Below we have included an update on our current findings.

In addition, we have also become interested in attempting to determine the mechanism(s) triterpenoids utilize to kill cancer cells when used as single agents. The mechanism of apoptotic induction by CDDO and derivates is somewhat controversial; however, evidence exists that caspase-8 is involved, thereby mimicking the effects of TNF-family death receptors [3]. Again, we have included below an update of our current findings when using triterpenoids as single agents.
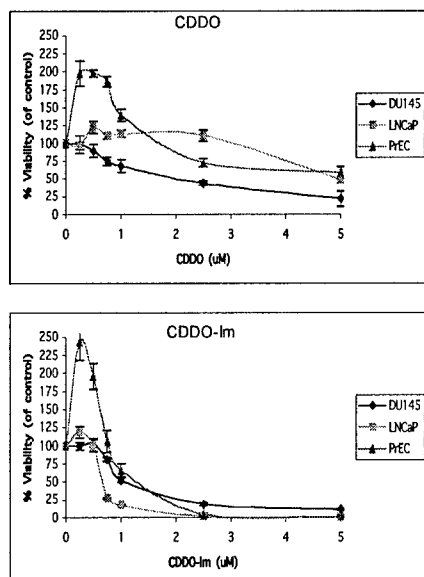
BODY:

Task 1. Task 1 was to identify potent triterpenoids that preferentially sensitize prostate cancer cells to apoptosis. In my studies, I have also included breast cancer cells because breast cancer cells displayed a more robust synergy response to triterpenoid and TRAIL combination treatment compared to prostate cancer cells.

a. To date, I have focused exclusively on CDDO and CDDO-Im because Michael Sporn (our collaborator at Dartmouth) informed me that CDDO-Me displayed poor pharmacokinetics *in vivo* (personal communication). CDDO and CDDO-Im induced apoptosis in both prostate cancer and normal prostate epithelial cells (PrEC) (Figures 1, 2). **Figure 1.** Cells (5.0×10^3/well) were seeded in 96 well plates. The next day cells were treated with CDDO and
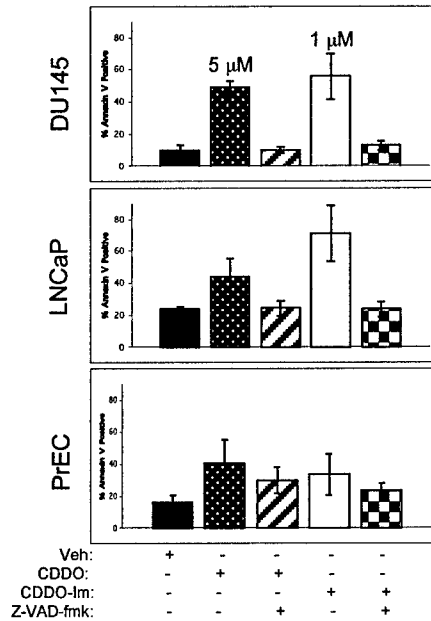
CDDO-Im. After 48 hours, the MTS cell viability assay was used to determine cell viability. CDDO and CDDO-Im-induced cell death was caspase-dependent because the pan spectrum caspase inhibitor, z-VAD-fmk, blocked cell death. **Figure 2 (left).** Cells (3.0×10^5/well) were seeded in 6-well plates. The next day cells were pre-treated for 1 hour with 100 μM z-VAD-fmk and then treated with CDDO or CDDO-Im. After 24 hours, cells were analyzed for annexin V/PI staining using a FACS Calibur Sorter. 10,000 events were sorted/ sample.

b.   Normal prostate epithelial cells (PrEC) appeared to be mildly sensitive to CDDO (5.0 μM) and CDDO-Im (1.0 μM) (Figures 1&2). However, I did not detect apoptosis in normal human breast epithelial cells (HMEC) when similar triterpenoid concentrations were used (Figure 1B appendix A and data not shown). Moreover, CDDO-Im (200 μg/day i.p. was well tolerated in mice (page 18 in appendix A).

c.   Using a combination of CDDO/CDDO-Im and TRAIL I detected synergy in both prostate and breast cancer cells (Figure 3 and Appendix A Figure 1B). **Figure 3 (below).** Cells (5.0×10^3/well) were seeded in 96 well plates. The next day cells were treated with CDDO/CDDO-Im in combination with 100 ng/ml TRAIL. After 48 hours, the MTS cell viability assay was used to cell viability.
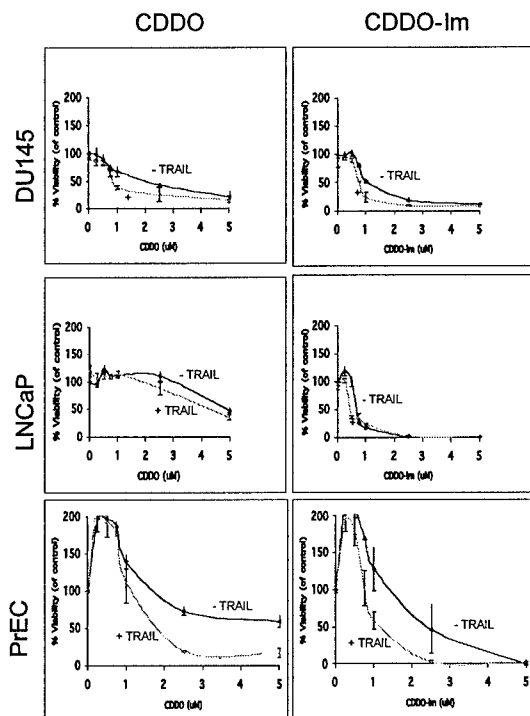

d.   I have not analyzed FLIP levels in LNCaP, DU145, and PrEC cells treated with triterpenoids. However, I have analyzed the effects of triterpenoids on FLIP in two breast cancer cell lines, MDA-MB-468 and T47D cells. Although both CDDO and CDDO-Im down-regulated FLIP in the breast cancer cells, CDDO was more efficient (Appendix A Figure 5).

   Task 2. Determine the mechanism(s) by which triterpenoids sensitize prostate and breast cancer cells to apoptosis.

a.   CDDO has been shown to sensitize PPC-1 prostate cancer cells to TRAIL even in the presence of PPARγ dominant-negative mutants [7]. These data indicate that CDDO functions through a PPARγ-independent mechanism.

b.   Inhibition of IKK and NF-κB activity by CDDO and CDDO-Me does not correlate with sensitization to TRAIL-induced apoptosis in PPC-1 cells [7]. Therefore, TRAIL sensitization by triterpenoids is likely not mediated by effects of NF-κB.

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c.   Several attempts were made to enucleate T47D breast cancer cells. Unfortunately these attempts were unsuccessful, in that the yield of cytoplast was very low, thwarting any attempt at cytoplast analysis. I suspect a similar problem will be encountered with the prostate cancer cells since the critical cytoplasm to nucleus ratio is similar in breast and prostate epithelial cells.
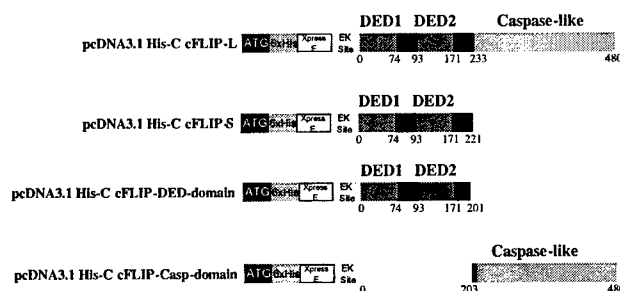
d.   Using the FLIP truncation mutants we attempted to identify the minimal FLIP domain necessary for CDDO-induced FLIP degradation. Unfortunately, all FLIP mutants were similarly degraded by CDDO suggesting perhaps that the minimal degradation domain was located in the caspase-like domain of FLIP. Therefore, we acquired a new set of truncation mutants, including a mutant containing only the caspase-like domain (**Figure 4 above**). PPC-1 cells were transfected with the FLIP mutants and the next day treated with or without CDDO (2.5 µM). After 24 hours, protein lysates were extracted and separated using SDS-PAGE, and then blotted onto nitrocellulose. **Figure 5 (right).** (A) An anti-HisG antibody was used to detect the FLIP truncation mutants 24 hours after CDDO treatment. (B) To confirm that CDDO would down-regulate $FLIP_L$ in PPC-1 cells, cells were treated for 24 hours with CDDO (0.5-5.0 µM).

Surprisingly, CDDO up-regulated the $FLIP_L$, $FLIP_S$, and DED mutants, yet down-regulated the caspase-like mutant. These data suggest that the minimal domain necessary for FLIP down-regulation is located within the first 201 amino acids. However, this data contradicts results obtained using our original FLIP truncation mutants, which suggested that the minimal degradation domain was located in the caspase-like domain. Further studies are necessary to resolve this conflict.
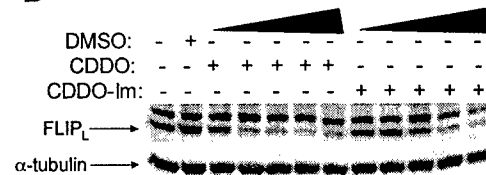

e.   We have not performed any mass spectrometry analysis.

f.   We have not performed any microarray experiments.
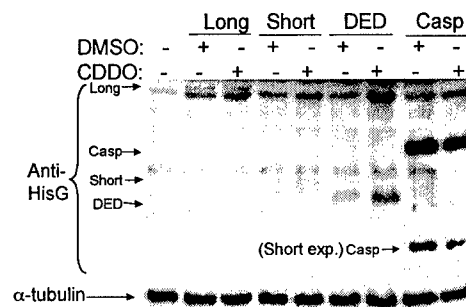
Task 3. Study the anti-tumor activity of triterpenoids and TRAIL in a mouse xenograft tumor model of prostate cancer. We have not performed any prostate xenograft studies to date, however we have tested the combination of CDDO-Im and TRAIL in a breast cancer tumor model. *In vivo* studies were performed using MDA-MB-468 breast cancer cells because triterpenoid and TRAIL synergy was superior to that observed in

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prostate cancer cells. The breast cancer data can be found in a paper submitted to the journal *Cancer Research* (Appendix A) in which I am the lead author. In brief, this paper demonstrates that combination CDDO-Im and TRAIL will reduce tumor burden in MDA-MB-468 xenograft tumor model; and demonstrates apoptosis in tumor tissue. Furthermore, this paper describes the lack of toxicity associated with CDDO-Im and TRAIL treatment *in vivo*.

One of the goals outlined in the SOW was to study triterpenoid and TRAIL synergy in prostate cancer cells. Although TRAIL and triterpenoid synergy was observed in prostate cancer cells (Figure 3), it was relatively modest compared to that observed in the breast cancer cells (Appendix A Figure 2). In lieu of this data, I believe that it would be worthwhile, at least in the prostate system, to focus on the mechanism triterpnoids utilize to induce apoptosis when used as a single agent, i.e. in the absence of TRAIL. Recall that triterpenoids, in particular CDDO-Im, were effective at inducing apoptosis in prostate cells when used as a single agent (Figure 3). With this in mind, it would be worthwhile pursuing the mechanism that CDDO and CDDO-Im utilizes to induce apoptosis in prostate cancer cells. Currently in the literature, there is controversy surrounding the mechanism triterpenoids utilizes to induces apoptosis. For example one report indicates that triterpenoids enter the apoptotic pathway through the extrinsic pathway [3] while another report contends that triterpenoids functions through the intrinsic pathway [2]. Since the mechanism of triterpenoid-induced apoptosis has not been evaluated in prostate cancer I believe it worthwhile to pursue this endeavor. I don't intend to completely abandon triterpenoid and TRAIL synergy studies in prostate cancer cells, however I would like to focus on using triterpenoids as a single agent in the prostate system. With this in mind, I have initiated the following studies below to identify the mechanism triterpnoids utilize to induce apoptosis in prostate cancer cells.

I have attempted to identify the order of caspase activation in CDDO and CDDO-Im treated DU145 and LNCaP cells using a time course study. **Figure 6.** DU145 and LNCaP cells were treated for the indicated times with either CDDO or CDDO-Im. Protein lysates were generated, separated using SDS-PAGE, and blotted onto nitrocellulose membrane. Membranes were probed with antibodies for PARP, caspase-8, -9, and α-tubulin. This stategy has not yet identified the order

of caspase activation however I will continue to pursue this strategy using antibodies for BID, cytochrome c, caspase-3, and –7. I will also generated DU145 and LNCaP stable cell lines expressing either CrmA to block the intrinsic pathway, or BcL-X_L to block the intrinsic pathway. Using these two strategies, should likely illucidate where triterpenoids enter the apoptotic pathway.
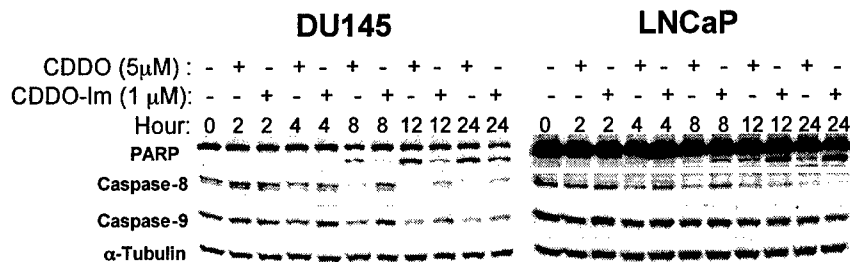
KEY RESEARCH ACCOMPLISHMENTS (for results using breast cells please see appendix A):

CDDO and CDDO-Im induce apoptosis in breast and prostate cancer cells.

CDDO and CDDO-Im sensitize breast and prostate cancer cells to TRAIL-mediated apoptosis.

CDDO and CDDO-Im down-regulated the anti-apoptotic protein c-FLIP$_L$.

CDDO and CDDO-Im up-regulate the cell surface death receptors DR4 and DR5.

CDDO-Im and TRAIL reduce tumor burden in a breast cancer xenograft tumor model.

CDDO-Im and TRAIL are well tolerated *in vivo* in mice.

REPORTABLE OUTCOMES:

Reportable research resulting from DOD funding includes the following:

1) Abstract entitled "Modulating TRAIL-mediated Apoptosis in Breast Cancer Cells Using the Synthetic Triterpenoids CDDO and CDDO-Im" presented at the American Association of Cancer Research 95nd Annual Meeting in Orlando, FL. (March 2004, Abstract # 5578). This presentation received an AFLAC Scholar in Training award.

2) Manuscript entitled "Synthetic Triterpenoids Cooperate with TRAIL to Induce Apoptosis of Breast Cancer Cells Both *in vitro* and *in vivo*" was submitted to the journal *Cancer Research*. This manuscript has been accepted pending revisions.

CONCLUSIONS:

We have identified a group of synthetic triterpenoids, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and its derivative 1-(2-cyano-3,12-dioxooleana-1,9-dien-28-oyl) imidazole (CDDO-Im), which induce apoptosis in breast and prostate cancer cells when used in the 1-5 μM range. Moreover, when used in the nanomolar range, these triterpenoids sensitize TRAIL-resistant breast cancer, and to a lesser extent prostate cancer cells, to TRAIL-mediated apoptosis.

In T47D and MDA-MB-468 breast cancer cells, we have demonstrated that TRAIL fails to initiate caspase-8 processing and consequently does not initiate TRAIL-mediated apoptosis. Concomitant treatment with CDDO or CDDO-Im reverses the TRAIL-resistant phenotype, leading to rapid induction of TRAIL-mediated apoptosis, while having no adverse effects on normal human mammary epithelial cells (HMEC). Mechanistically, CDDO and CDDO-Im 1) down-regulate the anti-apoptotic protein c-FLIP, which inhibits caspase-8 activation at the DISC (death-inducing signaling complex), 2) and induce up-regulation of the death receptors DR4 and DR5 on the cell surface. The combination of CDDO-Im and TRAIL reduces tumor burden in an in vivo MDA-MB-468 tumor xenograft model. After 14 days of combination CDDO-Im and TRAIL treatment, we found no significant toxicity in mouse tissues or hematological parameters.

Triterpenoid and TRAIL synergy in prostate cancer cells was less robust compared to that in breast cancer cells. However, triterpenoids will induce apoptosis in prostate cancer cells when used as a single agent (in the 1-5 μM range). With this in mind, I believe it would be worthwhile focusing on understanding the mechanism

triterpenoids utilize, when used as a single agent, to induce apoptosis in prostate cancer cells. In conclusion, triterpenoids used either alone, or in combination with TRAIL, represent a promising new cancer therapy, deserving further pre-clinical testing.

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Appendix A:

Synthetic Triterpenoids Cooperate with TRAIL to Induce Apoptosis of Breast Cancer Cells Both *in vitro* and *in vivo*

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Running Title: Cooperation of Synthetic Triterpenoids and TRAIL in Breast Cancer.

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Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL or Apo2L) has been shown to induce apoptosis specifically in cancer cells while sparing normal tissues. Unfortunately not all cancer cells respond to TRAIL; therefore, TRAIL sensitizing agents are currently being explored. We have identified a group of synthetic triterpenoids, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and its derivative 1-(2-cyano-3,12-dioxooleana-1,9-dien-28-oyl) imidazole (CDDO-Im), which sensitize TRAIL-resistant cancer cells to TRAIL-mediated apoptosis. Here we demonstrate that TRAIL-treated T47D and MDA-MB-468 breast cancer cells fail to process caspase-8 and consequently do not initiate TRAIL-mediated apoptosis. Concomitant treatment with CDDO or CDDO-Im reverses the TRAIL-resistant phenotype, leading to rapid induction of TRAIL-mediated apoptosis. From a mechanistic standpoint, we show that both CDDO and CDDO-Im down-regulate the anti-apoptotic protein c-FLIP$_L$, and up-regulate cell surface death receptors DR4 and DR5. Furthermore, CDDO and CDDO-Im, when used in combination with TRAIL, have no adverse affect on cultured normal human mammary epithelial cells (HMEC). Moreover, CDDO-Im and TRAIL are well-tolerated in mice and the combination of CDDO-Im and TRAIL reduced tumor burden *in vivo* in a MDA-MB-468 tumor xenograft model. These data suggest that CDDO and CDDO-Im may be useful for selectively reversing the TRAIL-resistant phenotype in cancer but not normal cells.

Introduction

TRAIL (TNF10, Apo-2L) is a member of the tumor necrosis factor (TNF) family of cytokines. TRAIL induces rapid apoptosis in many cancer cell types while sparing normal cells [1]. To date, five members of the human TNF Receptor superfamily have been identified that bind TRAIL. The death receptors DR4 (death receptor 4, TRAIL-R1, TNFR10A) [2] and DR5 (TRAIL-R2, TNFR10B) [3] contain conserved cytoplasmic death domains (DD) and are capable of binding TRAIL and initiating death signals. The decoy receptors DcR1 (TRAIL-R3, TNFR10C) [4] and DcR2 (TRAIL-R4, TNFR10D) [5] have close homology to the extracellular domains of DR4 and DR5, however, DcR1 lacks a transmembrane domain and DD, and DcR2 has a truncated, nonfunctional DD. Hence, both DcR1 and DcR2 bind TRAIL, but do not transmit death signals. Finally, TRAIL binds osteoprotegerin (OPG, TNFR11B) which is a soluble protein incapable of signaling [6].

Following TRAIL engagement with either DR4 or DR5, the ligated death receptors cluster and microaggregate within the cell membrane, thereby initiating formation of the death-inducing signaling complex (DISC) [7]. The functional DISC is composed minimally of death receptors (DR4 and DR5), adapter protein FADD, and caspase-8 or -10 (reviewed in [1] [8] [9]). Active caspases-8 and -10 cleave and activate downstream effector caspases (-3, -6, -7), which ultimately cut vital cellular substrates resulting in apoptosis (reviewed in [8]).

FLIP is an anti-apoptotic protein that has been detected in two isoforms, $FLIP_L$ (55 kDa) and $FLIP_S$ (28 kDa) [10]. Similar to pro-caspases-8 and -10, the FLIP proteins

contain a tandem pair of Death Effector Domains (DEDs), but they lack a catalytically active protease domain and thus can operate as trans-dominant inhibitors of caspases-8 and -10. During DISC formation, FLIP is preferentially recruited to the death receptor complex where it binds FADD and thwarts activation of caspase-8 and -10.

CDDO (2-Cyano-2-Dioxooleana-1,9,Dien-28-Oic Acid) [11] and the imidazole derivative CDDO-Im [12] are synthetic triterpenoids synthesized from the naturally occurring triterpene oleanolic acid. Both CDDO and CDDO-Im have been shown to suppress cellular proliferation and induce apoptosis in leukemia [13] [14], multiple myeloma [15], breast cancer [16], squamous cell carcinoma [17], and osteosarcoma [18] cells in culture. Previously CDDO has been shown to sensitize prostate, ovarian, colon [19], and leukemia [20] cells to TRAIL-mediated apoptosis. In the current report, we show that CDDO and CDDO-Im sensitize breast cancer cell lines to TRAIL-mediated apoptosis while having no effect on normal human mammary epithelial cells (HMEC). Moreover, CDDO and CDDO-Im down-regulate the anti-apoptotic protein $FLIP_L$ and up-regulate the death receptors DR4 and DR5 in breast cancer cells, rendering them sensitive to TRAIL. Finally, the combination of CDDO-Im and TRAIL is well tolerated in mice, and reduces tumor burden in a mouse xenograft model of breast cancer.

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Materials and Methods

Cell lines. T47D, PPC-1, OVCAR-3, PC-3M-LN4 [21], and LNCaP-LN3 [21] cells were cultured in RPMI 1640 (Irvine Scientific). Mouse embryonic fibroblasts, MDA-MB-468, and MCF-7 cells were cultured in DMEM with high-glucose (Irvine Scientific). RPMI and DMEM medium were supplemented with L-Glutamine (1.8 mM and 3.6 mM, respectively), Penicillin G (10,000 U/ml), Streptomycin Sulfate (10,000 µg/ml), and 10% heat-inactivated fetal bovine serum (Tissue Culture Biologicals, Tulare, CA). HMECs (Clonetics) were grown in MEGM® according to the manufacturer's instructions.

Production of Recombinant Soluble TRAIL. Competent BL-21 cells (Novagen) were transformed with pET15b plasmid (Novagen) containing a partial TRAIL cDNA encoding amino acids 95-281 with an inframe Flag and histidine$_6$ tag [22]. After inducing TRAIL expression by adding 2 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to bacteria in log-phase growth, the recombinant His$_6$-tagged protein was purified on Ni^{2+}-NTA columns under native conditions using the QIA*express* system (QIAGEN) as described previously [23]. Purified TRAIL was stored in aliquots at -80°C in 10% glycerol.

Cell viability assay. Cells (5.0×10^3-2.0×10^4) were seeded in 96 well plates and the next day (50-75% cell confluency) treated with various concentrations of CDDO, CDDO-Im, and TRAIL. Cytotoxicity was determined using the CellTiter96 AQ$_{ueous}$ one solution cell proliferation assay (Promega) according to the manufacturer's instructions. Plating cells at various dilutions confirmed assays were performed within the linear range of the assay.

For some experiments, the TRAIL-neutralizing antibody (clone 2E5, Abcam Limited, Cambridge, UK) was pre-incubated with recombinant TRAIL for 30 minutes prior to challenging cells. LNCaP LN3 cells were seeded on poly-lysine coated plates to maximize cell adherence.

Apoptosis assay. Cells ($5x10^5$) were seeded in 6 well plates and the next day treated with CDDO, CDDO-Im, TRAIL, DR4 mAb (TRAIL-R1), DR5 (TRAIL-R2), or various combinations of these reagents. After 12 or 24 hours, both adherent and floating cells were collected and stained with Annexin V and propidium iodide (PI) using the Annexin V-fluorescence isothiocyanate (FITC) apoptosis detection kit (Biovision) per the manufacturer's instructions. Ten thousand cells/treatment were analyzed using a flow cytometer (Becton Dickinson FACSort). TRAIL-R1 and TRAIL-R2 antibodies were kindly provided by Human Genome Sciences (Rockville, MD) [24].

Cell Surface DR4/DR5 quantification. Cells ($3.2x10^6$ T47D and $2.0x10^6$ MDA-MB-468) were seeded in 100 mm dishes and the next day treated with CDDO or CDDO-Im. After 18 hours, adherent cells were washed once with phosphate-buffered saline [pH 7.4], detached using a trypsin-free chelating solution [25], and re-suspended in ice-cold FACS buffer (3% heat inactivated FBS in PBS). Following centrifugation, cells were re-suspended in FACS buffer yielding 220,000 cells/50μl and incubated on ice for 15 minutes with 50 μg/ml human γ globulin (Cappel). Then cells were then incubated in the dark on ice with saturating concentrations of Phycoerythrin(PE)-labelled anti-DR4, anti-DR5, or IgG_1 isotype control antibodies (eBioscience) per the manufacturer's instructions.

After 1 hour, cells were washed once with FACS buffer and analyzed by flow cytometry. A total of 10,000 events were analyzed for each treatment.

RT-PCR. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed on total RNA prepared from T47D and MDA-MB-468 cells using the RNeasy Mini Kit (Qiagen). Primer sequences for each of the genes analyzed are as follows: DR4 forward primer: 5'-TGTTGTTGCATCGGCTCAGGTTGT-3', DR4 reverse primer: 5'-GAGGCGTTC CGTCCAGTTTTGTTG-3'; DR5 forward primer: 5'-GAGCGGCCCCACAACAAAAG AGGT-3', DR5 reverse primer: 5'-CAAGACTACGGCTGCAACTGTGAC-3', and GAPDH primers were purchased from Clontech. The linear range for GAPDH was determined to be between 20 and 30 cycles when 100 ng of total RNA was provided as template.

RT-PCR was performed using the SuperScript One-Step RT-PCR kit (Invitrogen), using 100 ng total RNA as template in each reaction. The thermocycler was programmed as follows: RT reaction, 50°C for 30 min.; post-RT denaturation: 94°C for 2 min.; 25 cycles of: 94°C for 30 sec.,54°C for 30 sec., 72°C for 45 sec.; elongation step: 72°C for 10 min., then samples were held at 4°C. The primer pairs for all genes were specifically selected such that all reactions could be performed simultaneously using a 54°C annealing temperature. RT-PCR products were analyzed using a 1.0% agarose gel and stained with ethidium bromide for visualization by UV-transillumination. Gels were imaged using a ChemiImager 4000 (Alpha Innotech) equipped with a multiImage light cabinet. Software from Alpha Innotech was used to quantify bands, normalizing data relative to GAPDH.

Immunoblotting. Breast cancer cells at 4×10^6/100 mm dish or HMEC at 1.5×10^6/100 mm dish were seeded and treated one day later with various agents (note: for DR4 and DR5 immunoblotting 3.2×10^6 T47D and 2.0×10^6 MDA-MB-468 cells were used, similar to FACS analysis). For caspase-independent experiments, cells were pre-treated for 1 hour with 100 µM z-VAD-fmk (MP Biomedicals). Cells were washed once with PBS, scraped into RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing a protease inhibitor cocktail (Sigma), incubated on ice for 30 min., passed 8 times through a 21 gauge needle, further incubated on ice 30 min., pelleted by centrifugation at 15,000xg for 20 min., and the supernatant was stored at -80 C. The DC Protein Assay (Bio-Rad) was used to determine protein concentrations. Cell lysates (35-75 µg) were subjected to SDS-PAGE (8-12% gels) and blotted onto 0.45 µm nitrocellulose membranes (Schleicher and Schuell). Membranes were probed with the following antibodies: 1:1000 (vol:vol) anti-FADD (Upstate), 1:1000 (v:v) anti-PARP clone C2-10 (BD Biosciences), 1:1000 (v:v) anti-Caspase-8 clone 5F7 (Upstate), 1:500 (v:v) anti-FLIP antibody Dave-2 (Alexis), 1:500 (v:v) anti-FLIP antibody NF6 (Alexis), 1:1000 (v:v) anti-TRAIL-R2 (Axxora), 1:500 (v:v) anti-DR4/TRAIL-R1 (Upstate), 1:2000 (v:v) monoclonal anti-α-Tubulin clone DM1A (Sigma), and 1:1000 (v:v) anti-BID (Cell Signaling). Secondary antibodies used were all HRP-conjugated (Amersham) and used at 1:2000 (v:v) dilution. Proteins were visualized using Super signal (Pierce) enhanced chemiluminescence detection substrate.

Tumor xenograft experiments. MDA-MB-468 cells (4.8×10^6) resuspended in 100 µl of serum-free DMEM were subcutaneously injected into the flanks of 4 week old female Balb c *nu/nu* mice using a 23 gauge needle. When tumor volumes reached 25 mm^3 (about 14 days), animals were treated daily for 14 days with intra-peritoneal (i.p.) injections of 100 µg/day of CDDO-Im [in 10% Cremephor-El (Sigma), 80% PBS, and 10% DMSO) then 6 hours later given i.p. injections of 5 mg/kg/day of TRAIL (in PBS). Tumor volume was measured every other day using vernier calipers and tumor volume calculated using the following formula: (long axis x short axis^2)/2. Each treatment group included 6 mice. Data were compared using a one-way analysis of variance (ANOVA).

Tissue and blood analysis. Mice were anaesthetized (n=3-5/group) using Avertin and blood collected via cardiac puncture. Serum chemistry and blood cell analysis was performed by the animal care program diagnostic laboratory at the University of California, San Diego (UCSD). Anaesthetized mice were then transcardinally perfused with ice-cold PBS [pH 7.4] for 2 min. followed by cold zinc-containing buffered formalin (Z-fix; Anatech. Inc.) for 5-10 minutes. After perfusion, tissues were immediately removed, post-fixed in Z-Fix, and embedded in paraffin. Dewaxed tissue sections (0.4 µm) were immunostained using a diaminobenzidine (DAB)-based detection method as described previously [26], employing the Envision-Plus-Horse Radish Peroxidase (HRP) system (DAKO) and using an automated immunostainer (Dako Universal Staining System). Polyclonal rabbit antiserum specific for the cleaved form of DNA fragmentation factor DFF40/CAD (ProScience M2007) was applied at 1:200 (v:v) dilution. The detection of nuclei with fragmented DNA by terminal deoxynucleotidyl

transferase [TdT] end-labeling was accomplished using The ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon) according to the manufacturer's instructions.

Statistical Analysis. Unless otherwise noted, data was analyzed using a nonparametric one-way ANOVA with a Bonferroni post-test. The confidence interval was set at 95%.

Results

CDDO and CDDO-Im sensitize breast cancer cells to TRAIL-induced apoptosis.

Here we examine a panel of breast, prostate, and ovarian cell lines for TRAIL sensitivity using an extracellular domain of recombinant soluble TRAIL (amino acids 95-281), which has been tagged with both FLAG and His [22]. In brief, cells were treated for 24 hours with TRAIL and then assessed for cell viability using the MTS assay. Consistent with previous reports [27], [19], [28], [29], PPC-1, OVCAR-3, and PC-3M LN4 cells were found to be TRAIL sensitive while LNCaP LN3, T47D, MCF-7, MDA-MB-468, and HMEC cells were TRAIL resistant (Figure 1A). To confirm the specificity of these results, we pre-incubated recombinant TRAIL with a TRAIL neutralizing antibody, which completely abrogated TRAIL-induced killing (data not shown). Thus, while some prostate and ovarian cancer lines are intrisically sensitive to TRAIL, 3 of 3 breast cancer lines were determined to be TRAIL resistant.

Previously, it was shown that CDDO and TRAIL cooperate to induce apoptosis of ovarian, prostate, and colon cancer cell lines [19]. Since breast cancer cell lines were found to be TRAIL-resistant, we sought to determine whether the synthetic triterpenoids, CDDO and CDDO-Im, would also cooperate with TRAIL to induce apoptosis of MDA-MB-468 and T47D breast cancer cells. Comparisons were made with normal mammary

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epithelial cells (HMEC). TRAIL-resistant cells were treated simultaneously with sub-toxic doses of either CDDO or CDDO-Im, in combination with low dose TRAIL ($\leq$ 250 ng/ml), and cell viability was determined after 24 hours. Both CDDO and CDDO-Im converted the TRAIL-resistant breast cancer cells to TRAIL-sensitive (Figure 1B). In contrast, CDDO and CDDO-Im did not sensitize normal human mammary epithelial cells (HMEC) to TRAIL-induced apoptosis (Figure 1B). Furthermore, by using a sequential treatment protocol, where cells were first treated with CDDO or CDDO-Im for 24 hours followed by TRAIL treatment, we were able to reduce the triterpenoid dose needed to sensitize breast cancer cell lines to the low nanomolar range (Figure 1C).

To confirm the cell death induced by the combination of triterpenoids and TRAIL was due to apoptosis, breast cancer cells were cultured with CDDO or CDDO-Im in combination with TRAIL and then assayed for apoptosis using Annexin V/PI staining. Using the accepted criterion that apoptotic cells are Annexin V-positive/PI-negative, we found that the combination of TRAIL and either CDDO or CDDO-Im induced both MDA-MB-468 and T47D breast cancer cells to undergo apoptosis at frequencies that were more than additive, compared to cells treated with TRAIL or triterpenoids individually (Figure 2). Apoptosis induced by the combination of TRAIL and triterpenoids was completely blocked when cells were pre-incubated for 30 minutes with 50 µM z-VAD-fmk (data not shown), a broad spectrum caspase inhibitor, thus confirming a caspase-dependent mechanism.

CDDO and CDDO-Im sensitize breast cancer cells to agonistic anti-DR4 and anti-DR5 monoclonal antibodies.

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TRAIL can stimulate apoptosis through either of the two death receptors, DR4 [2] or DR5 [3]. We explored the effects of CDDO and CDDO-Im on apoptosis induction of breast cancer cell lines using agonistic monoclonal antibodies that bind selectively to either DR4 or DR5. Like TRAIL, neither anti-DR4 or anti-DR5 induced significant amounts of apoptosis in cultures of MDA-MB-468 (Figure 3) or T47D (data not shown) breast cancer cells. In contrast, addition of CDDO or CDDO-Im to cultures sensitized in a concentration-dependent manner breast cancer cells to apoptosis induction by both anti-DR4 and anti-DR5, with DR4 more potent than DR5 (Figure 3).

TRAIL-mediated apoptosis is blocked upstream of caspase-8 in MDA-MB-468 and T47D cells.

To pinpoint the defect in TRAIL-mediated apoptosis in breast cancer cells, we analyzed: (a) caspase-8 cleavage, a proximal event in the TRAIL-induced apoptotic pathway; (b) FADD [30], an adapter protein bridging caspase-8 to DR4/DR5; and (c) BID [31] cleavage, a caspase-8 substrate, following TRAIL treatment of MDA-MB-468 and T47D cells. TRAIL treatment alone failed to induce caspase-8 and BID processing, or alter FADD levels, as did treatment with either CDDO or CDDO-Im (Figure 4). However, treatment with TRAIL in combination with either CDDO or CDDO-Im induced robust caspase-8 and BID processing. TRAIL and CDDO or CDDO-Im, in combination but not individually, also induced proteolytic processing of PolyADP Ribosylpolymerase (PARP), converting the 116-kDa protein to the 85-kDa form indicative of caspase-mediated cleavage (Figure 4). These data indicate that the block in

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the TRAIL-mediated apoptotic pathway occurs upstream or at the level of caspase-8 activation.

CDDO and CDDO-Im down-regulate FLIP$_L$ and up-regulate cell surface DR4 and DR5.

Previously we demonstrated that CDDO down-regulates the anti-apoptotic protein FLIP in prostate and ovarian cancer cells [19]. In the current report, we examined FLIP expression in breast cancer cells following CDDO and CDDO-Im treatment. Both CDDO and CDDO-Im induced dose-dependent reductions in the levels of FLIP$_L$ in MDA-MB-468 and T47D cells (Figure 5A). However, FLIP$_L$ down-regulation by CDDO was consistently more robust compared to CDDO-Im. Down-regulation of FLIP$_L$ by CDDO and CDDO-Im was caspase-independent because z-VAD-fmk failed to prevent triterpenoid-induced reductions in FLIP$_L$ (Figure 5B). Note that little FLIP$_S$ was detected in these breast cancer cells (data not shown) and the triterpenoids had no effect on the FLIP$_S$ levels.

Using FLIP *flip$^{+/-}$* and *flip$^{-/-}$* mouse embryo fibroblasts (MEFs) [32] we determined that FLIP status did not enhance the ability of CDDO and CDDO-Im to enhance TRAIL-mediated killing (data not shown). This finding suggested that triterpenoids enhance TRAIL killing through a FLIP-independent mechanism.

Therefore, we examined the effects of triterpenoids on cell surface expression of DR4 and DR5 in MDA-MB-468 and T47D, using flow cytometry. CDDO-Im increased cell surface DR4 and DR5 expression in a concentration-dependent manner on both MDA-MB-468 and T47D cells (Figure 5C). At equimolar concentrations, CDDO was

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less potent than CDDO-Im at altering cell surface DR4/DR5 levels, substantially increasing DR5 on T47D but not MDA-MB-468, and causing only a slight increase in DR4 expression on either MDA-MB-468 or T47D cells (Figure 5C).

Next, we analyzed DR4 and DR5 protein expression by immunoblotting using whole cell lysates to determine whether CDDO and CDDO-Im altered "total" DR4 and DR5 levels throughout the cell. CDDO-Im increased "total" DR5 protein levels in a dose-dependent manner (Figure 5D), consistent with the observed increased in DR5 on the cell surface. Note that the DR5 antibody used in these experiments recognized two DR5 splice variants. with approximate molecular weights of 46-kDa [$DR5_{S(Short)}$] and 52-kDa [$DR5_{L(Long)}$]. similar to previous reports [3] [1]. The increase in $DR5_S$ was more apparent compared to that of $DR5_L$ and densitometry analysis of the $DR5_S$ immunoblot results indicate that CDDO-Im increases $DR5_S$ protein levels by 2.0-3.5-fold relative to untreated cells (Figure 5D). In contrast to DR5, CDDO-Im did not detectably alter "total" DR4 levels throughout the cell (Figure 5D) despite increasing DR4 levels on the cell surface. With regard to DR4, these data suggest that CDDO-Im only induces DR4 redistribution to the cells surface. CDDO did not detectably increase "total" DR4 or DR5 when used at concentrations up to 1 μM (not shown), yet increased both DR4 and DR5 on the cell surface, suggesting that CDDO only redistributes DR4 and DR5 to the cell surface.

To determine whether CDDO-Im regulates DR5 gene expression at the mRNA level, we performed semi-quantitative RT-PCR to detect DR5 mRNA in both MDA-MB-468 and T47D cells following 16 hours of CDDO-Im treatment. Figure 5E shows that CDDO-Im increased DR5 mRNA levels in both of these breast cancer cell lines, with

MDA-MB-468 more affected than T47D. Image analysis of the ethidium-stained gels suggested that CDDO-Im induced a modest 25-75% increase in DR5 mRNA levels.

CDDO-Im cooperates with TRAIL to inhibit MDA-MB-468 xenograft tumor growth *in vivo*.

To determine whether CDDO-Im in combination with TRAIL could inhibit tumor growth *in vivo*, MDA-MB-468 tumor bearing mice were treated for 14 days with CDDO-Im (100 µg/day), TRAIL (5 mg/kg/day), the combination of CDDO-Im and TRAIL, or vehicle control. During the 14 days of treatment, the combination of CDDO-Im and TRAIL significantly inhibited tumor growth compared to either agent alone or vehicle control (Figure 6A). Upon treatment termination, tumors in the CDDO-Im and TRAIL treatment group resumed growth (data not shown), thus indicating the needed presence of CDDO-Im and TRAIL to maintain inhibition of tumor growth.

To explore the mechanism of tumor suppression, immunohistochemical analysis of caspase cleaved DFF40/CAD expression, TUNEL assay, and H&E staining were used to analyze resected tumors from mice following three consecutive days of treatment. On day four after treatment, tumors from mice treated with CDDO-Im and TRAIL contained numerous cells staining for cleaved DFF40/CAD (a marker of apoptosis), while only occasional immunopositive cells were found in vehicle-treated tumors (Figure 6B). Similarly, TUNEL-positive cells were numerous in TRAIL/CDDO-Im-treated tumors and confluent areas on necrosis were evident throughout tumors resected from these mice, compared to only occasional TUNEL positive cells in vehicle-treated mice and no evidence of necrosis (Figure 6B).

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Since data concerning CDDO-Im as a single chemotherapeutic agent is lacking, we treated MDA-MB-468 tumor-bearing mice for 14 days with high dose CDDO-Im (200 μg/day), observing no inhibition of tumor growth compared to the vehicle control (data not shown). These data emphasize the importance of including TRAIL in combination with CDDO-Im to achieve inhibition of tumor growth in this breast cancer model.

In vivo toxicity associated with CDDO-Im and TRAIL combination therapy was analyzed using several different toxicology parameters including animal weight change, behavior, blood chemistry, and tissue analysis. Animal weight changes were recorded over 14 days of combination CDDO-Im and TRAIL treatment and during 14 days of follow-up observation, and compared to weight changes associated with single agent and vehicle control treatments. The combination of CDDO-Im and TRAIL treatment resulted in significant weight loss by day three, however, by day twelve, body weight had returned and surpassed baseline weight; and there were no significant weight differences comparing the four treatment groups on day 15 (Figure 7A). Single agent treatment with either CDDO-Im or TRAIL did not induce significant weight loss. No significant weight changes were detected during the 14 day follow-up in any group.

Animal appearance and behavior (ruffled fur/lethargy) were observed over the 28 day experiment and no differences were noted comparing the different treatment groups.

H&E staining were also used to ascertain toxicity to mouse tissues (brain, liver, spleen, and kidney) following 14 consecutive days of CDDO-Im and TRAIL combination treatment, making comparisons with either agent alone and vehicle control treatments. **No staining differences were detected in brain, liver, spleen, and kidney tissues**

among treatment groups (Supplemental data Figure 1 shows examples of TUNEL staining of tissues following CDDO-Im and TRAIL combination treatment compared to vehicle control).

To further ascertain *in vivo* toxicity, we analyzed serum chemistries [sodium, potassium, alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, alkaline phosphatase (ALT), BUN, and Creatinine] in tumor-bearing mice on day 15 following 14 consecutive days of treatment. No significant differences were noted in means comparing the combination treatment group (CDDO-Im + TRAIL) to either single agent treatment or vehicle control (Figure 7B), suggesting that the combination of CDDO-Im and TRAIL is well tolerated *in vivo*.

The lack of significant TRAIL toxicity using native or "humanized" TRAIL is well documented in the literature (reviewed in [1] [33]), however much less is known about the *in vivo* toxicity of CDDO-Im. To determine whether higher CDDO-Im doses would be tolerated *in vivo*, tumor-bearing mice were treated for fourteen consecutive days with intraperitoneal injections of either vehicle, 150 μg CDDO-Im/day, or 200 μg CDDO-Im/day, and on day fifteen blood and tissue samples were collected for toxicology analysis. The same serum chemistry and tissue profiles described above were used for toxicology analysis, and additionally we counted the following in blood: hematocrit, hemoglobin, and red blood cells. CDDO-Im delivered at 200 μg/day for 14 days was well-tolerated in mice with the lone exception being mild anemia (RBC count 12.5% below normal and hematocrit 15.5% below normal, data not shown). No significant pathological observations were documented in the analyzed tissues. CDDO-Im caused a slight decrease in animal weight, peaking at day five, but weight loss returned to baseline

by the end of the treatment (data not shown). No changes in gross appearance or behavior (ruffled fur or lethargy) were noted over the fourteen day dosing schedule. These data suggest that CDDO-Im at doses of 150-200 µg daily are well-tolerated in mice.

Discussion

The data presented herein indicate that CDDO and CDDO-Im sensitize breast cancer cells to TRAIL-mediated apoptosis both *in vitro*. CDDO and CDDO-Im sensitization of tumor cells to TRAIL was associated with up-regulation of cell surface death receptors DR4 and DR5, and with down-regulation of the anti-apoptotic protein $FLIP_L$. CDDO/CDDO-Im-mediated reductions in $FLIP_L$ occurred via a caspase-independent mechanism, as shown by the failure of z-VAD-fmk to abrogate this effect. Furthermore, the combination of TRAIL and CDDO-Im were active at suppressing tumor growth *in vivo* in a tumor xenograft mouse model, while having little effect on these tumors individually. Toxicity *in vivo* of CDDO-Im either alone or in combination with TRAIL is minimal and overall the combination treatment is well-tolerated.

CDDO/CDDO-Im induced both down-regulation of $FLIP_L$ and up-regulation of cell surface DR4 and DR5 in breast cancer cells. Although both of these mechanisms may influence TRAIL sensitivity, the DR4/DR5 up-regulation seemed to correlate better with TRAIL sensitivity compared to a $FLIP_L$ down-regulation mechanism. For example in Fig. 1B there was no difference in cell viability between untreated and TRAIL treated T47D cells incubated with 0.75 µM CDDO or 0.25 µM CDDO-Im, yet these concentrations of triterpenoids were sufficient to down-regulate $FLIP_L$ (Fig. 5A). In

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contrast, CDDO/CDDO-Im uniformly up-regulated cell surface DR4 and/or DR5 in a dose-dependant manner (Fig. 5C) correlating nicely with TRAIL sensitivity (Figs. 1B). In addition, using FLIP $flip^{+/-}$ and $flip^{-/-}$ mouse embryo fibroblasts (MEFs) [32] we determined that FLIP status did not enhance the ability of CDDO and CDDO-Im to enhance TRAIL-mediated killing (data not shown). This finding suggested that triterpenoids enhance TRAIL killing through a FLIP-independent mechanism. Taken together, these data suggest that at least in some breast cancer cells triterpenoids likely sensitize to TRAIL mostly by up-regulating the death receptors DR4 and DR5.

In addition to analyzing cell surface DR4 and DR5 death receptors, we also examined the cell surface decoy receptors, DcR1 and DcR2, on T47D and MDA-MB-468 cells following CDDO and CDDO-Im treatments (data not shown). CDDO did not alter the number of decoy receptors in MDA-MB-468 and T47D cells. In contrast, CDDO-Im altered the number of decoy receptors in both cell lines. In T47D cells, CDDO-Im increased the number of DcR1 but decreased the number of DcR2 molecules on the surface, resulting in no net gain of surface decoy receptors. In MDA-MB-468 cells, although CDDO-Im increased both decoy receptors (DcR1 and DcR2) on the cell surface, the ratio of death receptors (DR4 and DR5) to decoy receptors (DcR1 and DcR2) remained in favor of death receptors (1.2:1.0). We conclude, therefore, CDDO-Im influences death receptor up-regulation to a greater extent than decoy receptor up-regulation, creating a more TRAIL-sensitive environment.

The mechanism by which CDDO and CDDO-Im alter cell surface DR4 and DR5 expression is currently unknown, however it might involve disruption of the intracellular redox balance leading to subsequent JNK activation. Recently, Yue et. al. found that

depletion of the anti-oxidant glutathione (GSH) contributed to JNK activation and subsequent DR5 up-regulation [34]. Moreover, it has been demonstrated that CDDO, CDDO-Me, and CDDO-Im increase intracellular reactive oxygen species (ROS) and decrease intracellular GSH levels leading to JNK activation [14]. Taken together, these findings raise the possibility that CDDO and its derivatives may up-regulate DR5 (perhaps also DR4) through effects on intracellular redox balance. Interestingly, the stability of the $FLIP_L$ protein is also know to be regulated by oxidative stress [35]. Thus, triterpenoid-induced oxidative stress could conceivably provide a unifying mechanism to explain both up-regulation of TRAIL receptors and down-regulation of FLIP.

We also examined the effects of CDDO and CDDO-Im on other anti-apoptotic proteins. For example, CDDO and CDDO-Im induced subtle XIAP down-regulation (data not shown); however, this down-regulation was completely abrogated using z-VAD-fmk, indicating the change was caspase-dependent. Therefore, triterpenoid-mediated XIAP down-regulation seems to be a consequence of sublethal caspase activation.

Little is known about the *in vivo* toxicity of CDDO-Im and no report exists using CDDO-Im in combination with TRAIL *in vivo*. We chose to analyze CDDO-Im and TRAIL toxicity in mice (on day four) following three consecutive days of CDDO-Im and TRAIL combination treatment. Day four was selected because animals in the combination CDDO-Im and TRAIL treatment group displayed maximal weight loss on day four and, therefore, we assumed toxicity would also be maximal at this time. Minimal alterations in serum chemistries, hematological parameters, and tissue samples were observed in mice treated with the combination of CDDO-Im and TRAIL,

suggesting that CDDO-Im and TRAIL were well-tolerated *in vivo*. Moreover, treatment with CDDO-Im alone, up to 200 μg/day for 14 days, was well-tolerated in mice. These pre-clinical toxicology data thus set the stage for more rigorous analysis of the combination of CDDO-Im and TRAIL in primates as an antecedant to human clinical trials.

CDDO-Im has also been used *in vivo* as a single agent to inhibit growth of B16 murine melanoma and L-1210 murine leukemia cancers, underscoring the potential use of CDDO-Im as a cancer therapeutic [12].

In summary, we have demonstrated that CDDO and CDDO-Im sensitize tumor cells but not normal cells to TRAIL-mediated apoptosis. Furthermore, CDDO-Im and TRAIL reduce tumor burden in a xenograft nude mouse model with minimal side effects. These findings underscore the potential for using synthetic triterpenoids as TRAIL sensitizers in cancer therapy.

Figure Legends

Figure 1. <u>Triterpenoids sensitize tumor cells to TRAIL.</u> (A) A panel of cell lines, both cancerous and normal, were screened for TRAIL sensitivity using Histidine$_6$-FLAG-tagged recombinant TRAIL. After 24 hours, the MTS assay was used to determine cell viability. (B) MDA-MB-468, T47D, and HMEC cells were treated simultaneously with either CDDO or CDDO-Im, in combination with 200 ng/ml TRAIL or vehicle, and then analyzed for cell viability after 24 hours. C) MDA-MB-468 cells were sequentially treated, first with CDDO/CDDO-Im for 24 hours, then with 100 ng/ml TRAIL for 24 hours. Data represent mean ± standard error (n=3) and are representative of three independent experiments.

Figure 2. <u>Combined treatment with triterpenoids and TRAIL induces apoptosis of breast cancer cells.</u> MDA-MB-468 and T47D cells were treated with either CDDO (0.5 μM for MDA-MB-468 and 0.85 μM for T47D cells) or CDDO-Im (0.5 μM), in combination with 100 ng/ml TRAIL, and assayed for apoptosis after 12 hours by the Annexin V/PI staining. Data are representative of three independent experiments.

Figure 3. <u>Triterpenoids sensitize breast cancer cells to anti-DR4 and anti-DR5 monoclonal antibodies.</u> MDA-MB-468 cells were treated with either (A) CDDO or (B) CDDO-Im in combination with TRAIL (100 ng/ml), DR4 Ab (5000 ng/ml), DR5 Ab (5000 ng/ml), or DR4 and DR5, and stained for Annexin V/PI after 24 hours. Data are representative of three independent experiments.

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Figure 4. <u>Triterpenoids collaborate with TRAIL to induce proteolytic processing of</u> <u>caspase-8, BID, and PARP.</u> The effects of CDDO (0.7 µM) and CDDO-Im (0.7 µM), with and without TRAIL (200 ng/ml) on FADD, caspase-8, BID, and PARP were evaluated by immunoblotting in T47D and MDA-MB-468 cells. Both adherent and floating cells were collected for analysis after18 hours of treatment. For PARP, the black arrow indicates the 116 kDa full length PARP and the white arrow indicates the 85 kDa cleaved fragment.

Figure 5. <u>CDDO and CDDO-Im down-regulate FLIP$_L$ and up-regulate cell surface</u> <u>DR4 and DR5 in MDA-MB-468 and T47D cells.</u> (A) Cells were treated for 18 hours with increasing concentrations of CDDO or CDDO-Im. Lysates were prepared, normalized for total protein content, and analyzed by SDS-PAGE/immunoblotting using anti-FLIP and anti-Tubulin antibodies. (B) Cells were pre-treated for 1 hour with 100 µM z-VAD-fmk prior to CDDO and CDDO-Im treatment for 18 hours (CDDO concentrations used=0.4 µM for MDA-MB-468 and 1.0 µM for T47D; CDDO-Im concentrations used=0.5 µM for MDA-MB and 1.0 µM for T47D cells). Cell lysates were prepared and analyzed by immunoblotting. (C) Cell surface DR4 and DR5 expression was measured by flow cytometry on MDA-MB-468 and T47D cells following CDDO or CDDO-Im treatment for 18 hours using anti-DR4 and anti-DR5 antibodies conjugated to Phycoerythrin (key: DMSO vehicle treatment with isotype matched control antibody-*shaded no line*; DMSO vehicle with either DR4 or DR5 staining-*thin line*, triterpenoid with either DR4 and DR5 staining-*thick line)*. Bar graph data indicate fold

increase in mean fluorescence intensity of DR4 and DR5 surface expression relative to vehicle control. Triterpenoid concentrations used range from 0.25-1.0 μM (except for CDDO treated T47D cells where 0.5-2.5 μM was used). Data are representative of two independent experiments. (D) Immunoblotting was used to quantify total DR4 and DR5 protein levels in whole cell lysates following 18 hours of CDDO-Im treatment. Graph (bottom) indicates relative $DR5_s$ expression (normalized to both loading and vehicle controls), as measured by scanning densitometry. (E) Semi-quantitative RT-PCR was used to quantify the DR5 mRNA levels following 16 hours of CDDO-Im treatment (normalized to both loading and vehicle controls).

Figure 6. <u>CDDO-Im in combination with TRAIL inhibits MDA-MB-468 tumor growth in nude mice and histological analysis of xenograft tumors.</u> (A) Animals bearing pre-established tumors (n=11 per group) were dosed daily for 14 days with i.p. injections of CDDO-Im (100 μg/day) in the morning and TRAIL (5 mg/kg/day) in the afternoon. On day one, tumor volume was measured prior to treatment. Data points shown indicate mean ± SEM. Mean tumor volumes were considered statistically significant ($^*p < 0.001$, $^{\dagger}p < .01$, $^{+}p < 0.05$). (B) MDA-MB-468 tumors from mice treated for three consecutive days with i.p injections of either CDDO-Im (100 μg/day)+TRAIL (5 mg/kg/day) or vehicle+vehicle control were analyzed histologically. H&E (20x), TUNEL (20x), and cleaved DFF40/CAD (40x) staining were used to detected apoptosis and necrosis. TUNEL and DFF40/CAD staining are brown and TUNEL-stained tumors were counter stained using methyl green, while DFF40/CAD

stained sections were counterstained with hematoxylin. Shown are representative fields from 3 analyzed animals/treatment.

Figure 7. <u>Toxicology analysis of combination treatment with CDDO-Im and TRAIL.</u> *In vivo* toxicity associated with CDDO-Im and TRAIL treatment was assessed in tumor-bearing mice treated with combination CDDO-Im (100 µg/day) and TRAIL (5 mg/kg/day), either agent alone, or vehicle control. (A) Animal weights shown were recorded during the 14 days of treatment. Weight on day 1 was obtained prior to initiation of treatment. Means on day 3 were statistically significant [+]$p<0.05$, [‡]$p<0.001$. (B) Animals were treated for 14 consecutive days as indicated above and on day 15 the following blood chemistry parameters were analyzed: sodium, potassium, ALT, AST, alkaline phosphatase, BUN, creatinine, and glucose. Data displayed indicate the mean ± standard error. There were no statistical differences in mean values.

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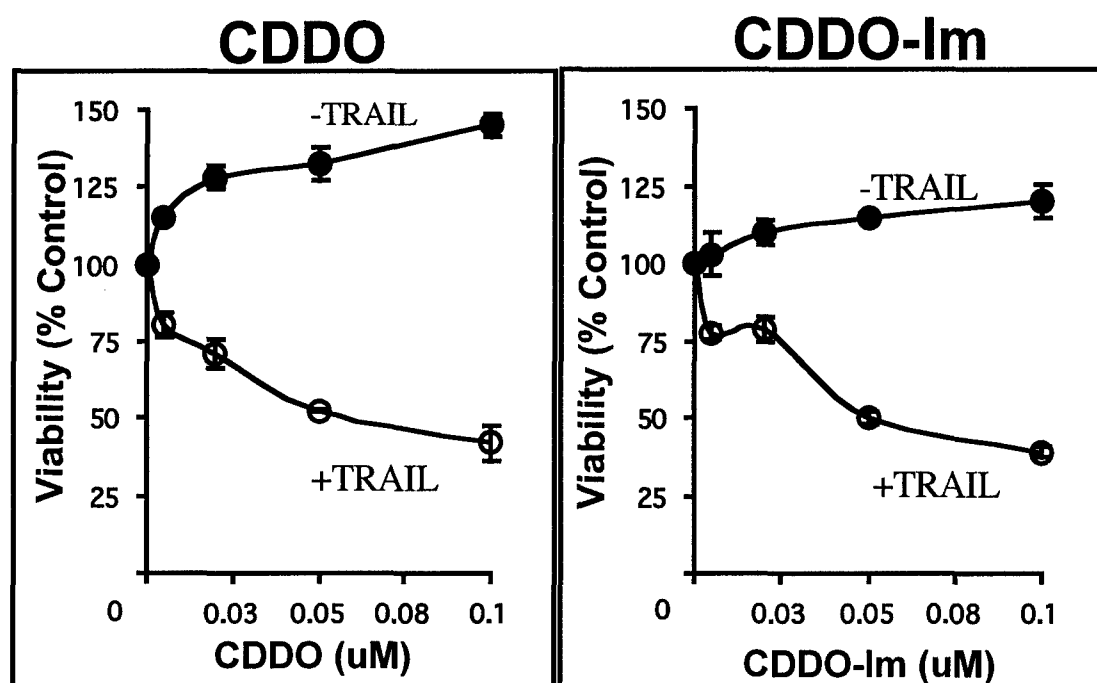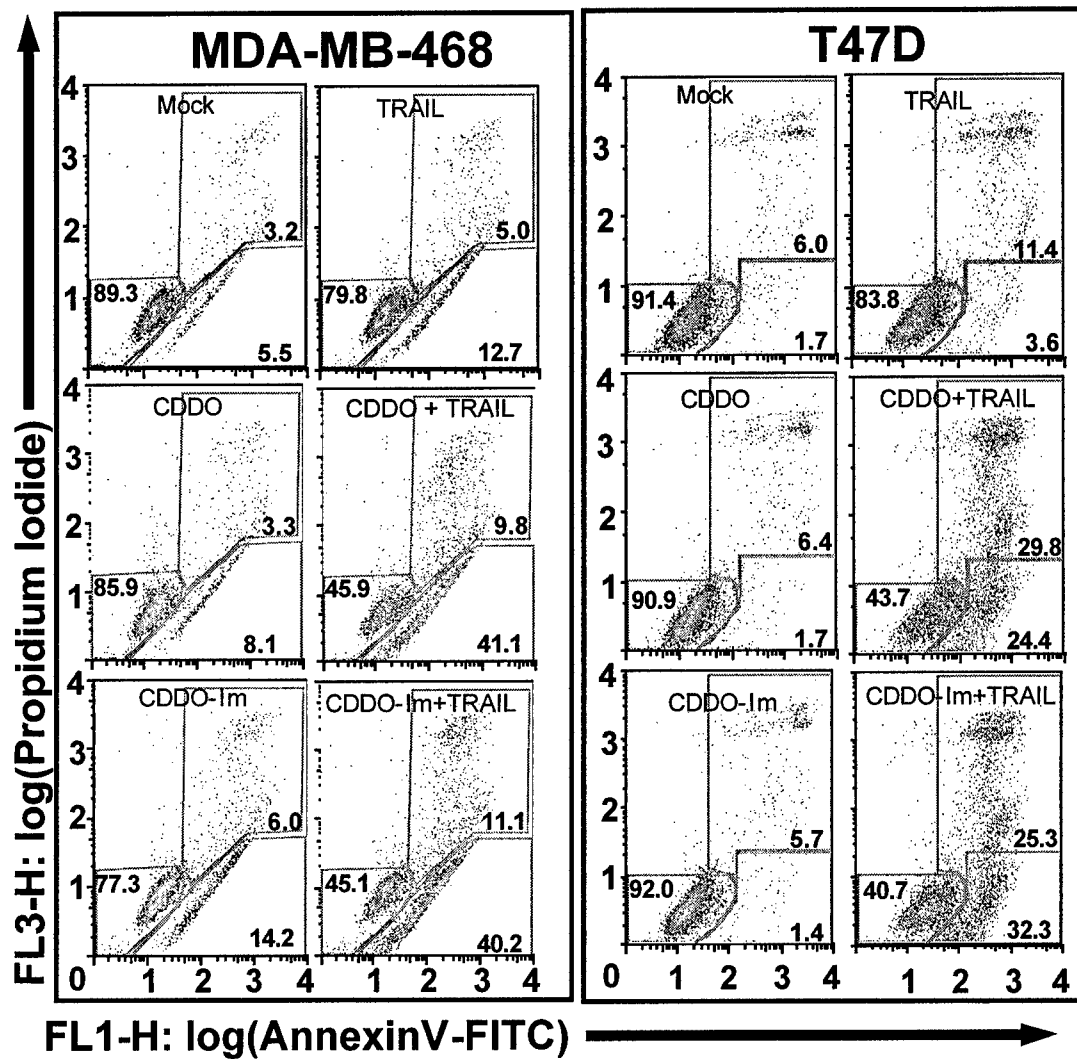
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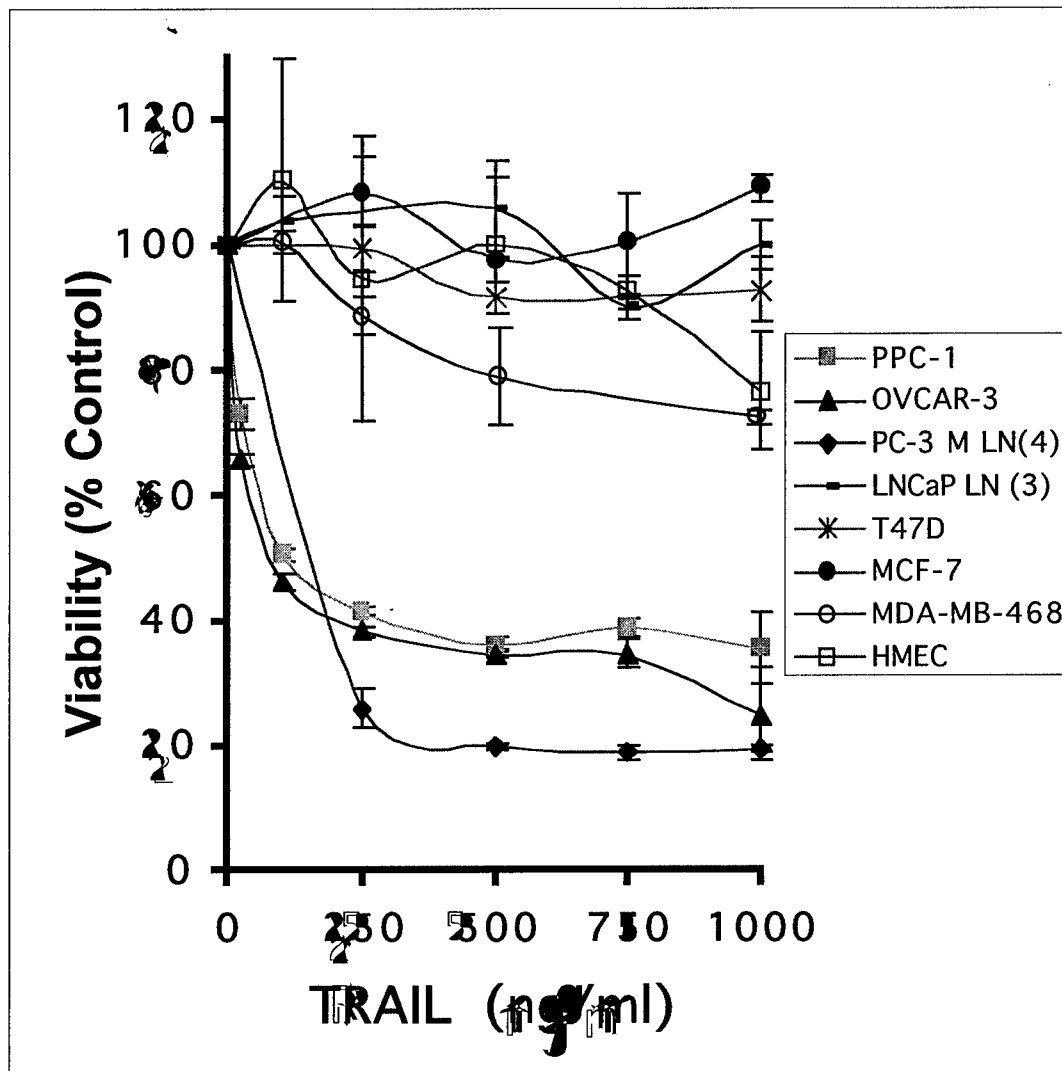
Figure 1B
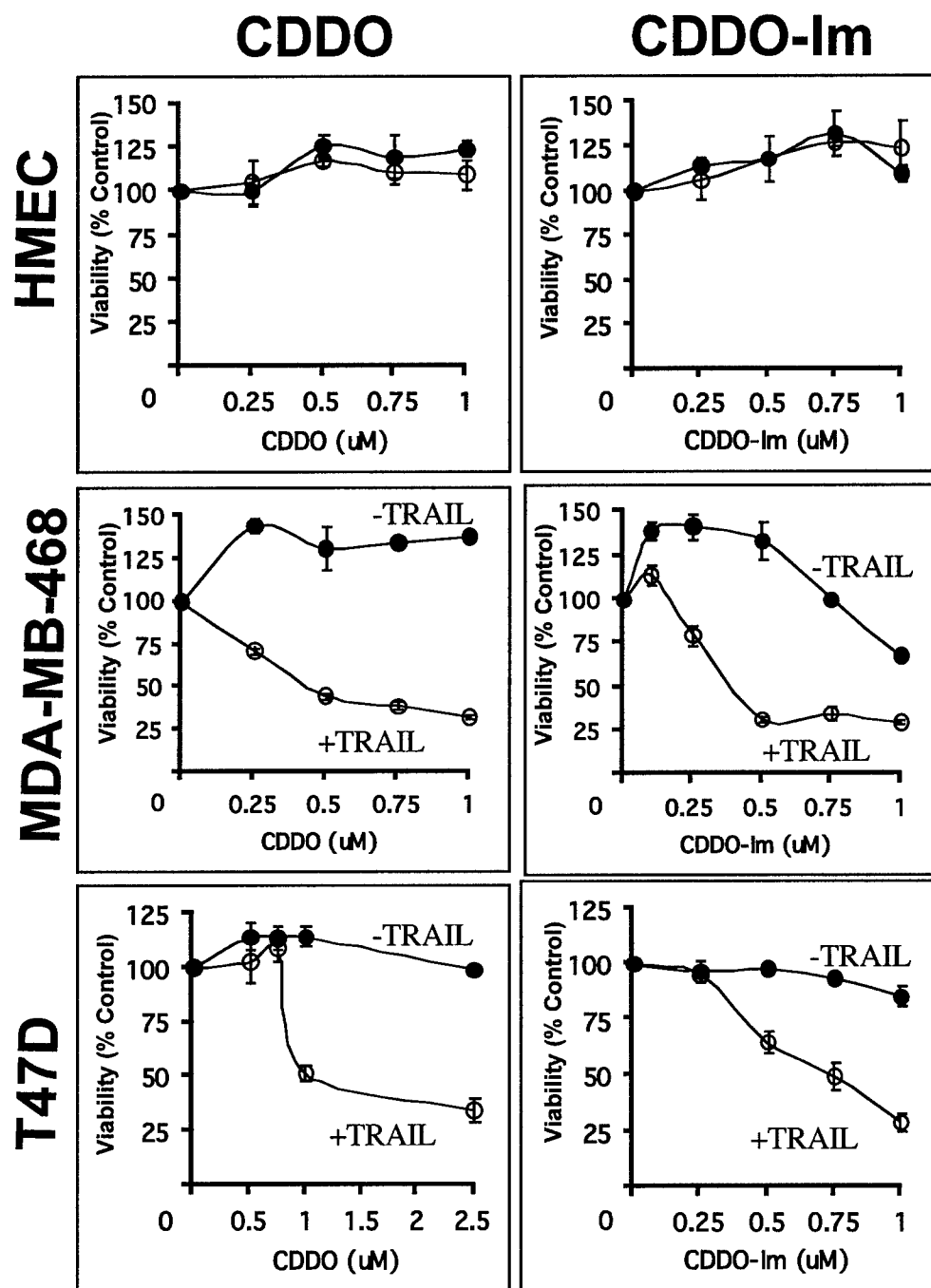
Figure 1C

Figure 2

Figure 3

Figure 4

MDA-MB-468 **T47D**

TRAIL	:	-	+	-	+	-	+	-	+	-	+	-	+
CDDO	:	-	-	+	+	-	-	-	-	+	+	-	-
CDDO-Im	:	-	-	-	-	+	+	-	-	-	-	+	+

FADD ▶

Caspase-8 ▶

BID ▶

PARP ▶
▷

α-Tubulin ▶

Figure 5

A

MDA-MB-468   **T47D**

CDDO(μM):   Veh   0.25   0.5   0.75   1.0   Veh   0.75   1.0   2.5

FLIP$_L$ ▶

α-Tubulin ▶

CDDO-Im(μM):   Veh   0.25   0.5   0.75   1.0   Veh   0.25   0.5   0.75   1.0

FLIP$_L$ ▶

α-Tubulin ▶

B

MDA-MB-468   **T47D**

CDDO:	-	-	+	+	-	-		-	-	+	+	-	-
CDDO-Im:	-	-	-	-	+	+		-	-	-	-	+	+
Z-VAD-FMK:	-	+	-	+	-	+		-	+	-	+	-	+

FLIP$_L$ ▶

α-Tubulin ▶

Figure 5C

Figure 5D&E

D

MDA-MB-468

CDDO-Im (µM): 0, Veh., 0.25, 0.5, 0.75, 1.0

DR5$_L$ ▶
DR5$_S$ ▶

DR4 ▶

α-tubulin ▶

T47D

CDDO-Im (µM): 0, Veh., 0.25, 0.5, 0.75, 1.0

DR5$_L$ ▶
DR5$_S$ ▶

DR4 ▶

α-tubulin ▶

E

MDA-MB-468

Veh., 0.25, 0.5, 0.75, 1.0

DR5

GAPDH

T47D

Veh., 0.25, 0.5, 0.75, 1.0

DR5

GAPDH

Relative DR5$_S$ protein vs CDDO-Im (uM)
- MDA-MB-468
- T47D

Relative DR5 mRNA vs CDDO-Im (uM)
- MDA-MB-468
- T47D

Figure 6A

Figure 6B

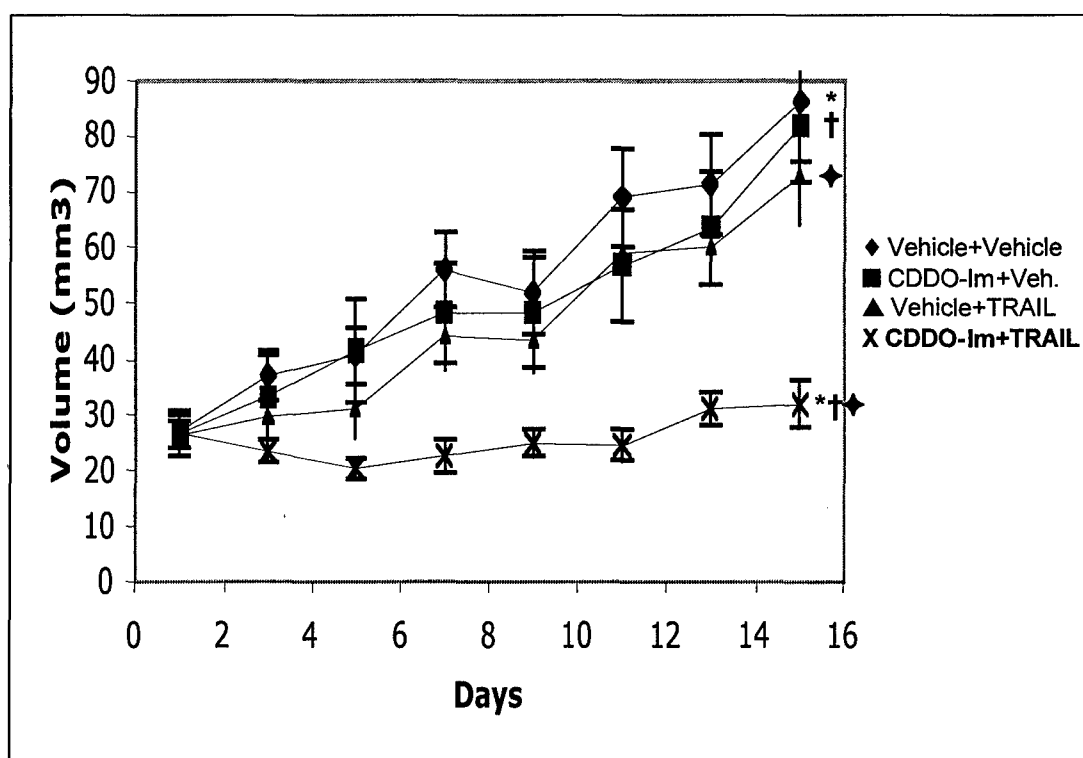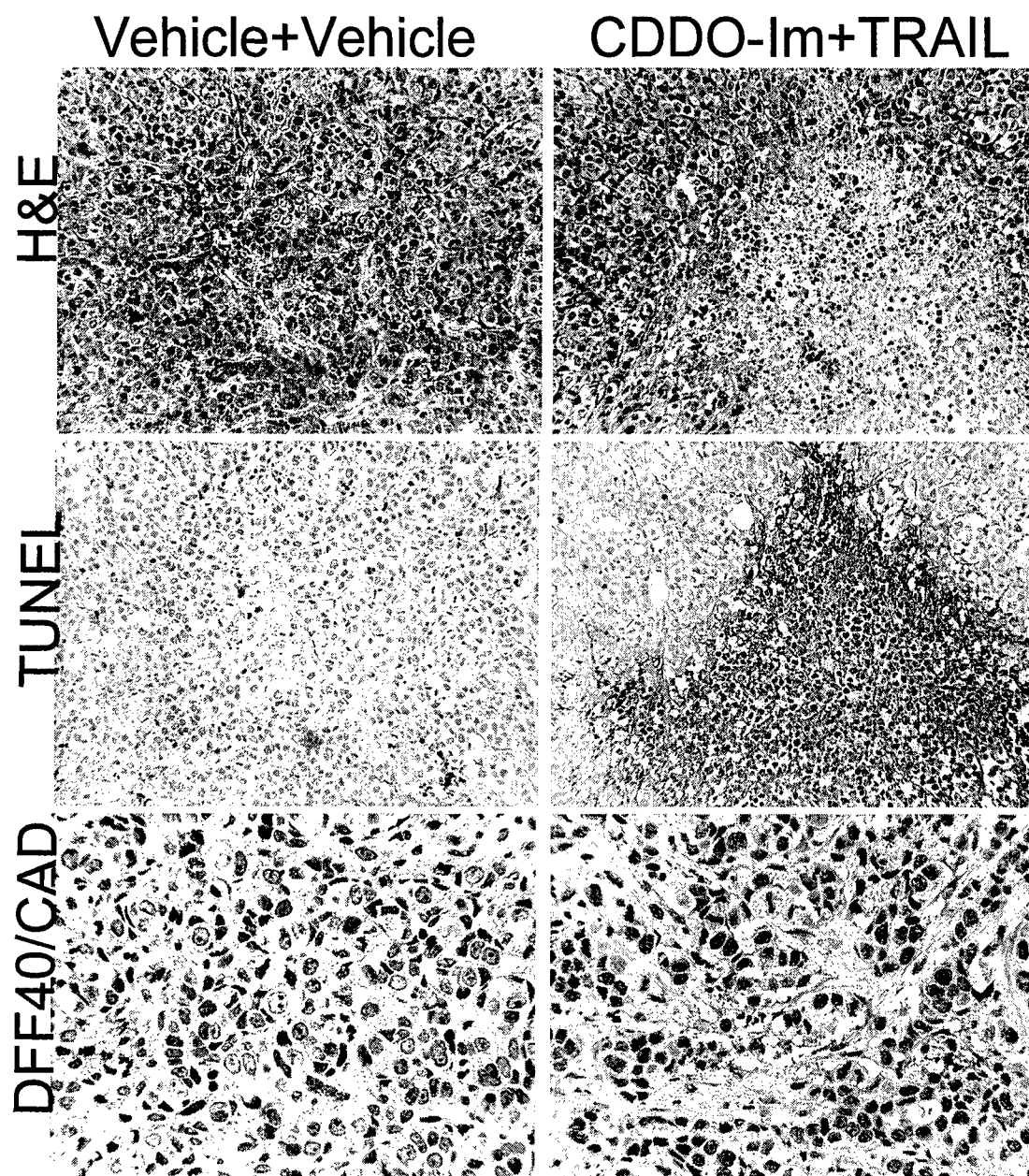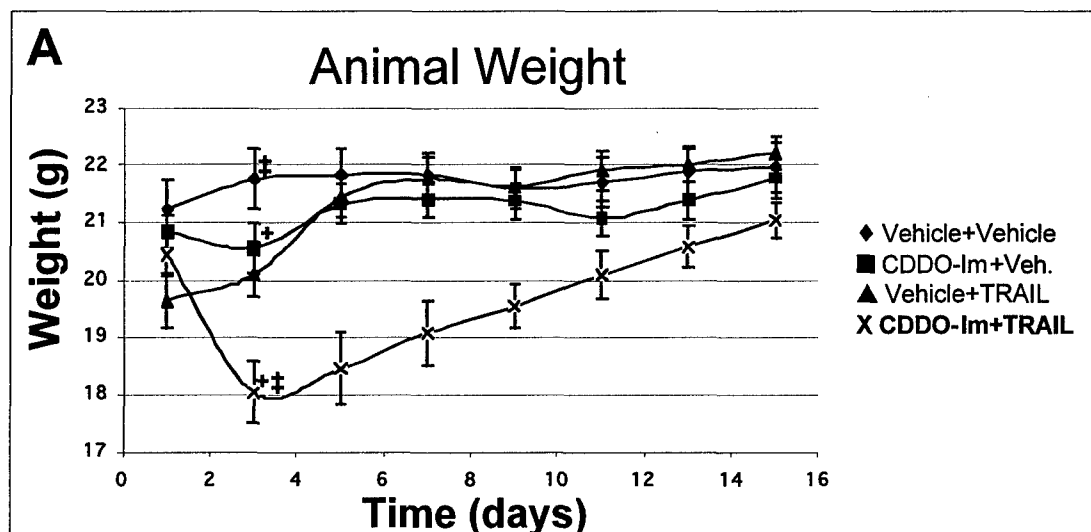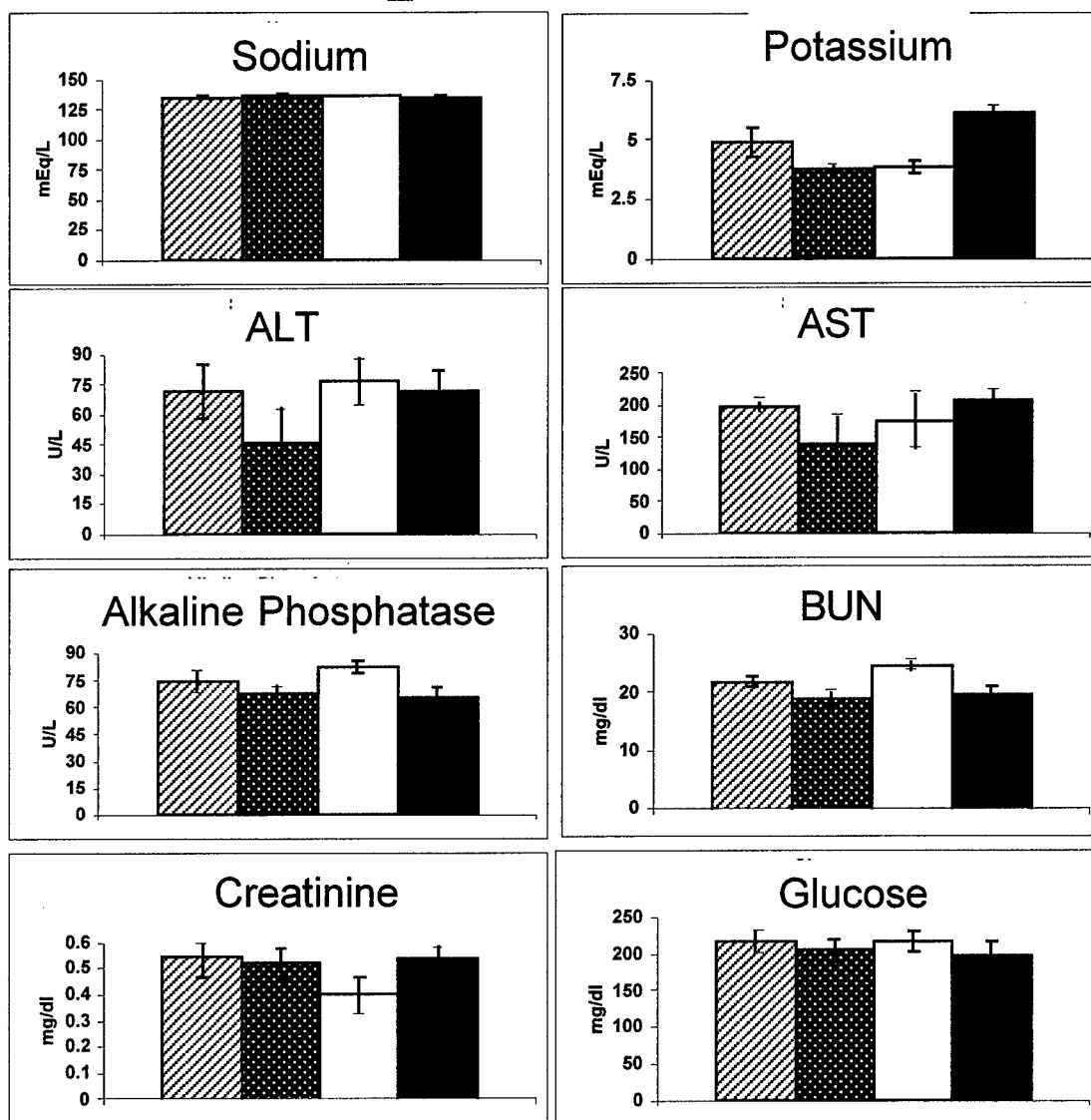|	Vehicle+Vehicle	CDDO-Im+TRAIL
H&E		
TUNEL		
DFF40/CAD		

Figure 7

Figure 7B

Key:
- ▨ Vehicle + Vehicle
- ▦ CDDO-Im + Vehicle
- ☐ Vehicle + TRAIL
- ■ CDDO-Im + TRAIL

Supplemental Figure 1

	Vehicle+Vehicle	CDDO-Im+TRAIL
Liver		
Kidney		
Spleen		

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